

AWARD NUMBER: W81XWH-15-1-0223

TITLE: Do Androgen Receptor Splice Variants Facilitate Growth of Bone Metastases?

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REPORT DATE: November 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
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1. REPORT DATE November 2016		2. REPORT TYPE Final		3. DATES COVERED 1 Sep 2015 - 31 Aug 2016
4. TITLE AND SUBTITLE Do Androgen Receptor Splice Variants Facilitate Growth of Bone Metastases?			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER W81XWH-15-1-0223	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Nancy L. Weigel, Ph.D. E-Mail: nweigel@bcm.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, TX 77030			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)	
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Among the mechanisms for resistance to anti-androgen therapy is expression of constitutively active AR splice variants, which lack the carboxyl terminal hormone binding domain. The best characterized variant is AR-V7. Expression of this variant is especially prominent in bone metastases and the morbidity and mortality due to bone metastases is one of the most significant problems in the treatment of PCa. The role of variants in PCa is still contentious. We have used lentiviruses to make LNCaP and VCaP cell lines that express AR-V7 in response to doxycycline and have compared gene expression regulated by AR and AR-V7 in the LNCaP lineage. We found many differences between the two isoforms. One of the most striking was evidence of activation of Notch signaling by AR-V7. Notch signaling has been implicated in growth of bone metastases. We hypothesize that AR-V7 mediated induction of Notch signaling to promotes growth of bone metastases. Our initial studies show that AR-V7 induces a number of bone related genes, which may facilitate growth in a bone microenvironment.				
15. SUBJECT TERMS Androgen receptor, splice variant, bone				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 7
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified		
				19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION: Prostate cancer (PCa) is an androgen dependent disease and the treatment for metastatic PCa is androgen deprivation therapy (ADT). Tumors become resistant to both first and second line ADT, but there is good evidence that many of them remain androgen receptor (AR) dependent. Among the mechanisms of resistance is expression of constitutively active AR splice variants, which lack the carboxyl terminal hormone binding domain. Of these, the most common, best characterized variant is AR-V7, which contains exons 1-3 of AR and 16 unique amino acids derived from a cryptic exon. Expression of this variant is especially prominent in bone metastases and the morbidity and mortality due to bone metastases is one of the most significant problems in the treatment of PCa. The role of variants in PCa is still contentious. Whether they can substitute for AR and/or have unique functions has not yet been resolved. We have used lentiviruses to make LNCaP and VCaP cell lines that express AR-V7 in response to doxycycline and have compared gene expression regulated by AR and AR-V7 in the LNCaP lineage. We found many differences between the two isoforms. One of the most striking was evidence of activation of Notch signaling by AR-V7. Notch signaling has been implicated in growth of bone metastases. We hypothesize that AR-V7 mediated induction of Notch signaling to promote growth of bone metastases. We will determine whether expression of AR-V7 promotes differentiation of cells in osteogenic medium, alters differentiation of bone cells, and facilitates growth of bone metastases. We will utilize a series of in vitro assays to assess whether the expression of AR-V7 causes the LNCaP (and VCaP) cells to mimic the actions of the C4-2B cells, a line derived originally from LNCaP cells, which produces osteoblastic lesions in vivo. Second, we will determine whether induction of AR-V7 in LNCaP and VCaP cells increases growth of tumors in an intratibial injection model. If the hypothesis is correct, we will determine whether inhibition of Notch signaling counteracts these actions and, in the long term, this will provide additional rationale to seek inhibitors of variant expression/activity.

2. KEYWORDS androgen receptor, splice variant, bone, C4-2B, Notch

3. ACCOMPLISHMENTS.

Specific Aim 1: To determine whether expression of AR-V7 induces an osteomimetic phenotype in differentiating medium and causes secretion of factors that induce differentiation of bone cells to osteoblasts.

Task 1: Test cells for capacity to mineralize and markers of mineralization. These studies were based on the assays and findings of Lin et al. (2001) The Prostate 47:212-233. We proposed to use the C4-2B and MC3T3 cells used in this paper as positive controls and then to test effects in the LNCaP/LNCaP AR-V7 and VCaP/VCaP/AR-V7 models. The reported calcium assay kit from Sigma is no longer available and there was insufficient information to determine whether they were measuring total or free calcium. In replicating their alizarin red assays for calcium retention, we found that their extraction method was very weak and their data were normalized, so we could not assess whether we were extracting equivalent amounts of alizarin red. Thus, we tested a number of methods in the literature and have found that a cetyl pyridinium assay is more sensitive and quantitative. We have obtained a no cost extension to finish these studies and the comparison of the cell lines currently is in progress. The authors also reported that several bone genes increased somewhat when C4-2B cells were grown in pro-mineralization medium. When we blasted the reported primers, we found that they also had other targets. Thus we designed series of primers and tested them against mRNA isolated from a commercially available osteoblast cell pellet. Once we had established a good series of primers, we compared expression in MC3T3, C4-2B, LNCaP, and VCaP cells in control and pro-mineralization medium. Although we didn't see much response of the C4-2B cells, the VCaP cells (isolated from a bone metastasis) showed a remarkable induction of the target genes in response to the mineralization medium (Fig. 1) (see appendix). To our knowledge, this has never been tested. Next, we sought to determine whether AR-V7 altered expression of these genes. Cells were transferred to charcoal stripped serum and treated with doxycycline to induce AR-V7.

Although there wasn't much effect of the pro-mineralization medium, in each cell line pair, expression of AR-V7 increased target gene expression (Fig 2). Charcoal stripping removes hormone, but also many other factors. We plan to retest the effects of AR-V7 in the complete medium to determine whether effects would be higher and/or more dependent on the mineralizing medium.

Task 2: Test cells for capacity to induce osteoblastic differentiation of MC3T3-E1 cells.

Specific Aim 2: To determine whether induction of AR-V7 in LNCaP and VCaP cells increases growth of tumors in an intratibial injection model. As described in our animal protocol, the first task was to use subcutaneous tumors to determine whether chow containing doxycycline would induce AR-V7 in the tumors. Tumors were established (LNCaP and LNCaP Ar-V7) and animals fed the doxycycline containing chow. AR and AR-V7 were assayed by western blotting. We found no induction of AR-V7. Thus, we tested doxycycline (0.2 mg/ml in 5% sucrose) in the water. This regime revealed induction of AR-V7 in the LNCaP AR-V7 cells with no animal weight loss (doxycycline is bitter and without sucrose mice often fail to drink sufficient water and begun dehydrated). Thus, we have established appropriate conditions for the LNCaP series. Our VCaP series requires more DOX to induce, so we are rederiving the line to obtain one that can be induced at the same level of DOX as LNCaP. We will be performing the LNCaP/LNCaP AR-V7 study as planned and the VCaP/VCaP AR-V7 study providing the new line is sufficiently responsive to doxycycline.

Training opportunities: N/A

Dissemination of results: Nothing to report

4. IMPACT Our initial results suggest that AR-V7 induces changes consistent with growth of bone metastases. The studies in the no-cost extensions period will further test this hypothesis.

Impact on other disciplines: nothing to report

Impact on technology transfer: nothing to report

Impact on society: nothing to report

5. Changes/problems. As described in accomplishments, we have had to change many of the conditions for the assays, but the overall goals remain unchanged.

6. PRODUCTS nothing to report

7. Participants : Nancy Weigel unchanged

Jianghua Wang unchanged

William Bingman unchanged

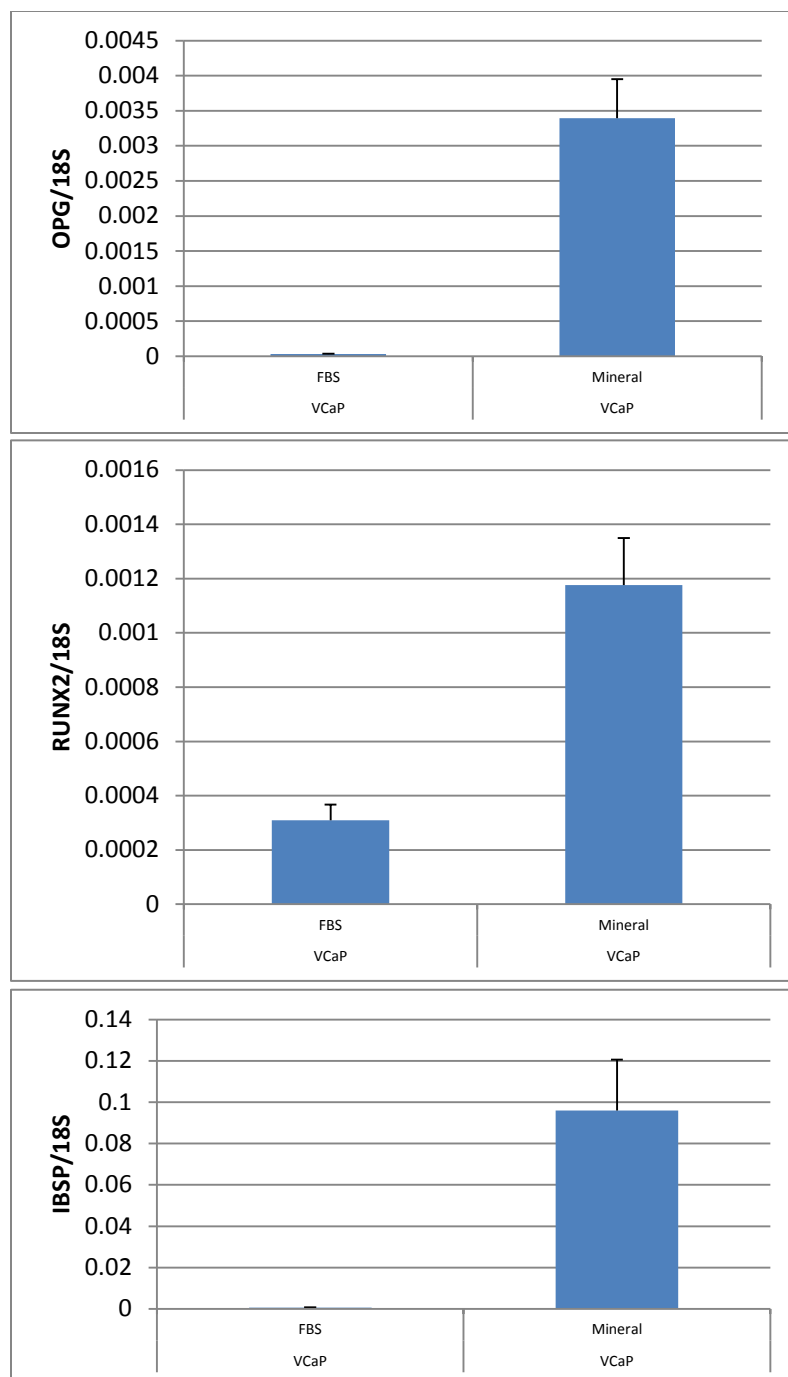


Figure 1. Comparison of bone gene mRNA levels in control medium and in mineralizing medium. VCaP cells were plated in 6 well plates and transferred to fresh medium +/- mineralizing treatment (10 nM beta glycerol phosphate and 50 ug/ml ascorbic acid) the following day. Cells were harvested after 72 hours, RNA purified and expression levels for osteoprotegrin (OPG), RUNX2 (Cbfa1) and IBSP (BSP) measured by qRT-PCR and normalized to 18S RNA. In each case AR-V7 increased expression of target genes.

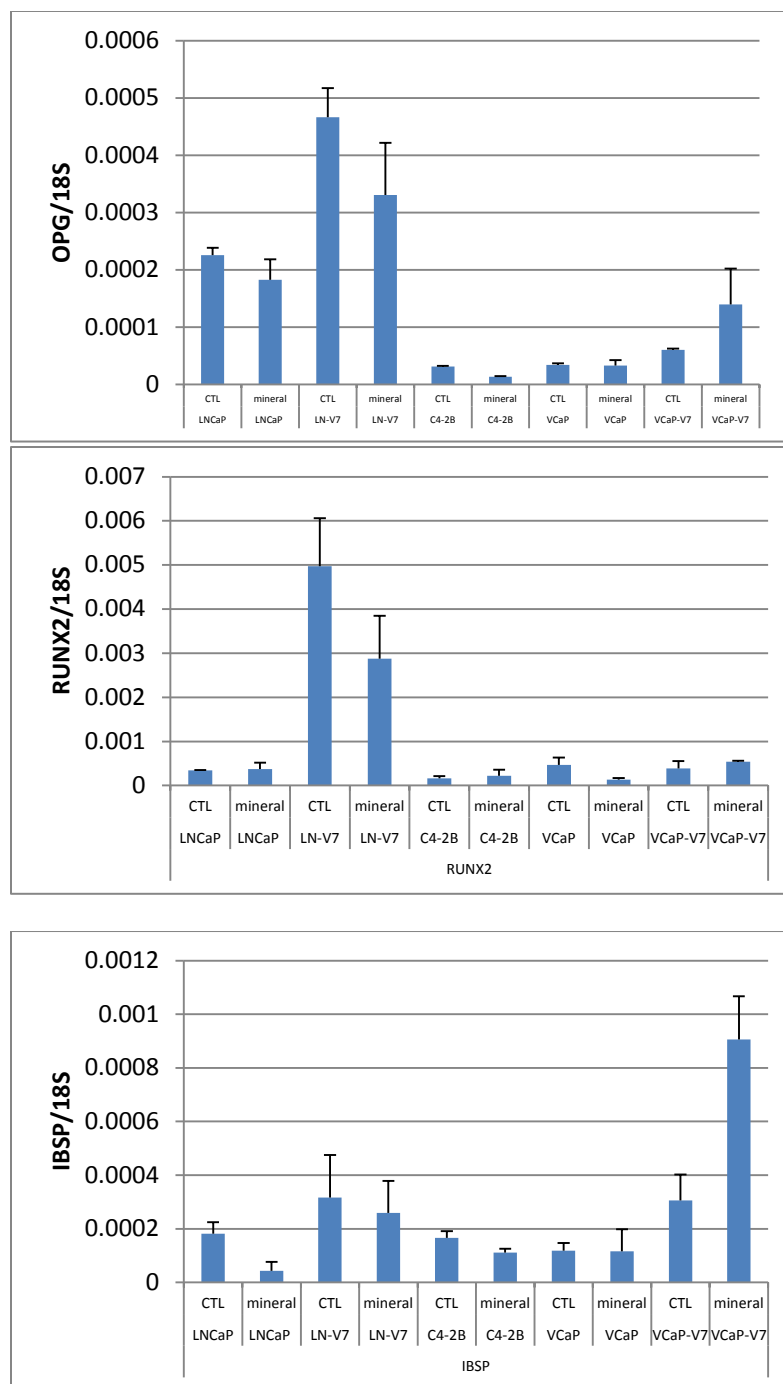


Figure 2. Comparison of bone gene mRNA levels in control medium and in mineralizing medium. Cells were plated in 6 well plates and transferred to medium containing charcoal stripped serum +/- mineralizing treatment (10 nM beta glycerol phosphate and 50 ug/ml ascorbic acid) the following day and 20 ng/ml doxycycline added to LNCaP AR-V7 cells and 300 ng/ml doxycycline to VCaP AR-V7 cells. Cells were harvested after 72 hours, RNA purified and expression levels measured by qRT-PCR and normalized to 18S RNA. In each case AR-V7 increased expression of target genes.